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Insights into Fern Evolution from Mapping Chloroplast Genomes

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ABSTRACT.—Within the leptosporangiate ferns, chloroplast DNA (cpDNA) structural mutations have been identified by physical mapping. Earlier work revealed that members of four disparate "higher" fern families all possess cpDNAs that share the same set of complex rearrangements in the inverted repeat. *Osmunda* cpDNA lacks these rearrangements. We have examined the chloroplast genomes of additional ferns and determined that *Polypodium* and the schizaeaceous ferns share the rearrangements previously found in *Cyathea, Pteridium, Adiantum,* and *Polystichum.* Two additional cpDNA structural types also have been identified. A partial duplication and relocation of the gene *chlL* usually occurs with the other gene rearrangements found in "higher" ferns but may be lacking in the heterosporous water fern, *Marsilea.* Also, a structural type lacking any of these complex rearrangements, but still different from *Osmunda,* occurs in *Gleichenia* cpDNA. The phylogenetic implications of these findings conflict, at least in part, with previously proposed evolutionary hypotheses.

Numerous pteridologists have offered detailed hypotheses of fern evolutionary relationships (reviewed in Smith, 1995; Pichi Sermolli, 1974). These scenarios, for the most part, have been intuitive attempts at generating phylogenetic hypotheses. Unfortunately, there is little consensus on the basic structure of the proposed trees (Fig. 1). Searching for markers to characterize major lineages, we have examined chloroplast genome structure in some ferns representating groups commonly regarded as pivotal in the analysis of higher-level fern phylogeny.

Land plant chloroplast genomes share many features of basic organization, gene content and gene order (Palmer, 1991). Although instances of structural change are relatively uncommon (reviewed in Downie and Palmer, 1992; Palmer, 1991; Palmer et al., 1988), where present these mutations can serve as important phylogenetic markers. For example, the lycopsids, *Lycopodium, Selaginella,* and *Isoetes,* share with the bryophytes the same orientation of a 30-kb region. All other vascular plant cpDNAs, including *Psilotum, Equisetum,* and the eusporangiate ferns *Botrychium* and *Marattia,* possess the derived, inverted gene order (Raubeson and Jansen, 1992a) indicating that the lycopsids are the basal extant lineage of vascular land plants. Other examples of phylogenetically-informative structural changes include an inversion marking the Barnadesiinae as the basal lineage of Asteraceae (Jansen and Palmer, 1987), a loss of one copy of the IR linking members of a subtribe of legumes (Lavin, Doyle, and Palmer, 1990), and a separate IR loss event supporting the monophyly of conifers (Raubeson and Jansen, 1992b).

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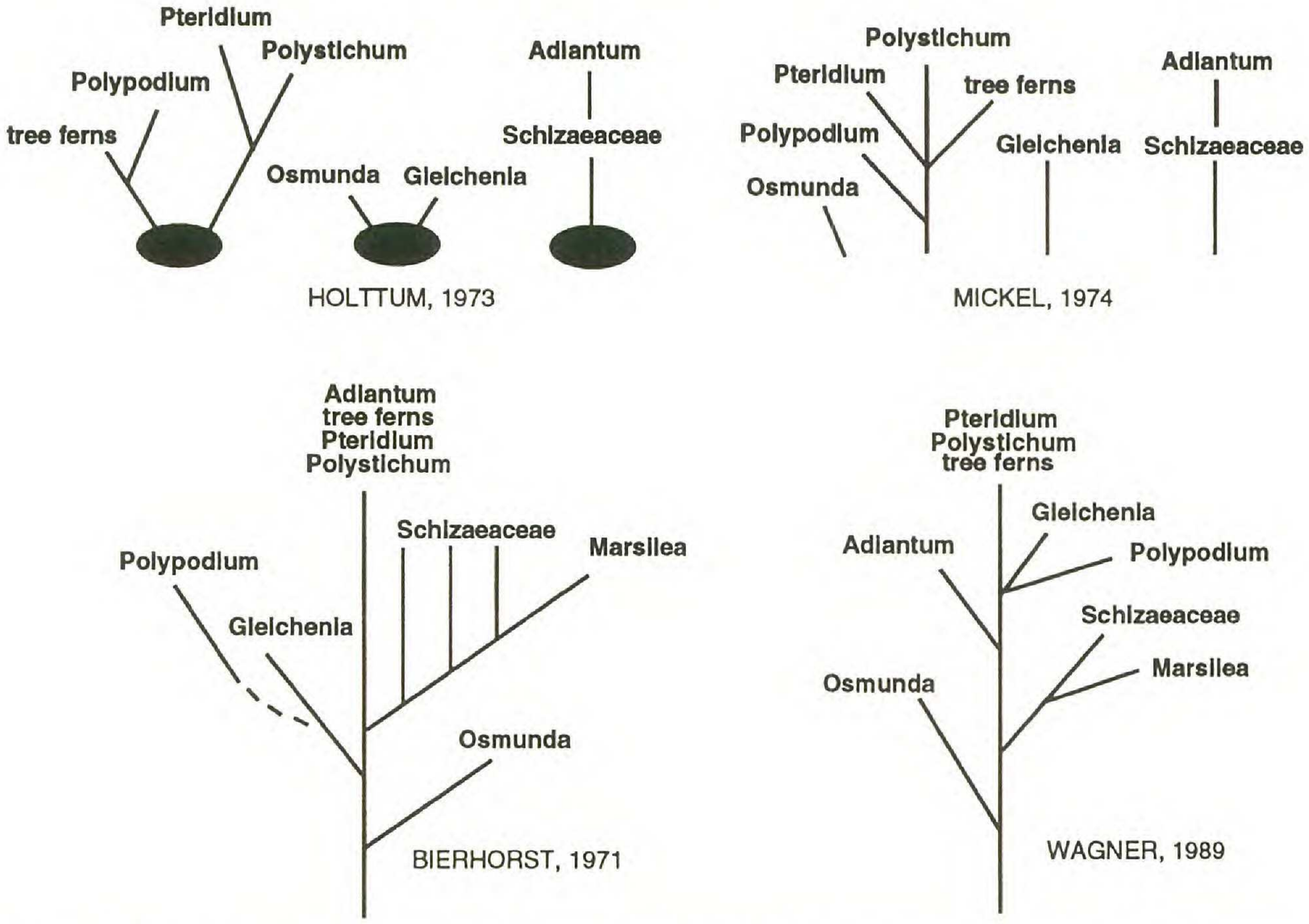

FIG. 1. Schematic representations of four influential phylogenetic scenarios. The phylogenies have been simplified to include only those taxa discussed in this paper.

Structural rearrangements also exist in the chloroplast genomes of ferns. Recently *Adiantum capillus-veneris* cpDNA was cloned and characterized (Hasebe and Iwatsuki, 1990). Stein et al. (1992) determined that structural changes found in *Adiantum* are shared by additional leptosporangiate ferns: *Pteridium, Polystichum* and *Cyathea.* These changes include gene duplications that occurred through the addition of genes formerly found in the large single copy (LSC) region to the inverted repeat (IR). In addition, inversions in the IR changed the orientation of the rDNA operon and moved the newly duplicated *psbA* gene to the opposite end of the IR, close to the small single copy (SSC) region. Although these four taxa represent a small sampling of leptosporangiate ferns, the implication of finding the derived mutations in members of four disparate families (but not in *Osmunda*), is at odds with many published hypotheses of fern relationships, such as those (Fig. 1) of Holttum (1973) and Mickel (1974), which postulate multiple lineages of ferns arising from a "pre-*Osmunda*" ancestor.

Shared structural mutations have the potential to delimit phylogenetic groups and clarify aspects of fern evolutionary history. Therefore, we have characterized the cpDNA structure of additional ferns (Table 1). These ferns represent groups that are putatively more derived than *Osmunda,* but still potentially basal to those previously studied.

TABLE 1. Fern cpDNAs examined for rearrangements. Absence of a particular rearrangement or set of rearrangements indicated by 0; presence by +.

Taxon	*ndhB* duplication	*psbA* plus two inversions	*chlL* partial duplication[1]
Osmunda cinnamomea[2]	0	0	0
Gleichenia bancroftii	+	0	0
Marsilea quadrifolia	+	+	0
Anemia phyllitidis	+	+	+
Lygodium palmatum	+	+	(+)
Schizaea robusta	+	+	+
Polypodium aureum	+	+	(+)
Adiantum capillus-veneris[3,4]	+	+	+
Polystichum acrostichoides[4,5]	+	+	+
Dryopteris ludoviciana[6]	+	+	+
Pteridium aquilinum[4,7]	+	+	(+)
Sphaeropteris elongata[8]	+	+	+
Dicksonia gigantea[9]	+	+	(+)

[1]For this column, + indicates presence of both the partial duplication and the movement of the duplicated portion away from the SSC/IR junction, (+) indicates presence of the partial duplication but that the position of the duplicated portion could not be determined.
[2]Palmer and Stein (1982), Raubeson (1991).
[3]Hasebe and Iwatsuki (1990).
[4]Stein, et al. (1992).
[5]Burke et al. (1993).
[6]Hutton (1992).
[7]Tan and Thomson (1990).
[8]All other members of the Cyatheaceae that have been mapped, plus *Lophosoria quadripinnata,* also show this pattern; see Conant et al. (1994), although only *Sphaeropteris* possessed restriction sites to determine that the duplicated part of *chlL* was also relocated.
[9]Stein, Conant and Valinski (in press).

MATERIALS AND METHODS

We characterized cpDNA structure by preparing physical maps via overlap hybridization (Palmer, 1986). Total genomic DNA was prepared from fresh leaf tissue by one of two methods: 1) modified CTAB extraction (Doyle and Doyle, 1987) with two percent wt/vol PVP (polyvinylpyrrolidone, MW 40,000) added to the extraction buffer; or 2) extraction via organellar isolation (Stein, 1993) in a buffer containing PEG (polyethylene glycol, MW 3500). Restriction digests (single and double digests of PstI, PvuII, StuI, and SphI), agarose gel electrophoresis, membrane transfers and Southern hybridizations were performed as described in Raubeson (1991) and Stein (1993). Probes in the hybridizations were prepared from cloned tobacco (Olmstead and Palmer, 1992) and *Adiantum* (Hasebe and Iwatsuki, 1990) cpDNA fragments, and portions of the *chlL* gene were PCR-amplified from *Polystichum acrostichoides* DNA. These PCR products (261-bp and 363-bp) are completely internal to *chlL* and share their 3′ terminus; the 363-bp fragment contains more of the 5′ end of the gene (details in Burke et al., 1993).

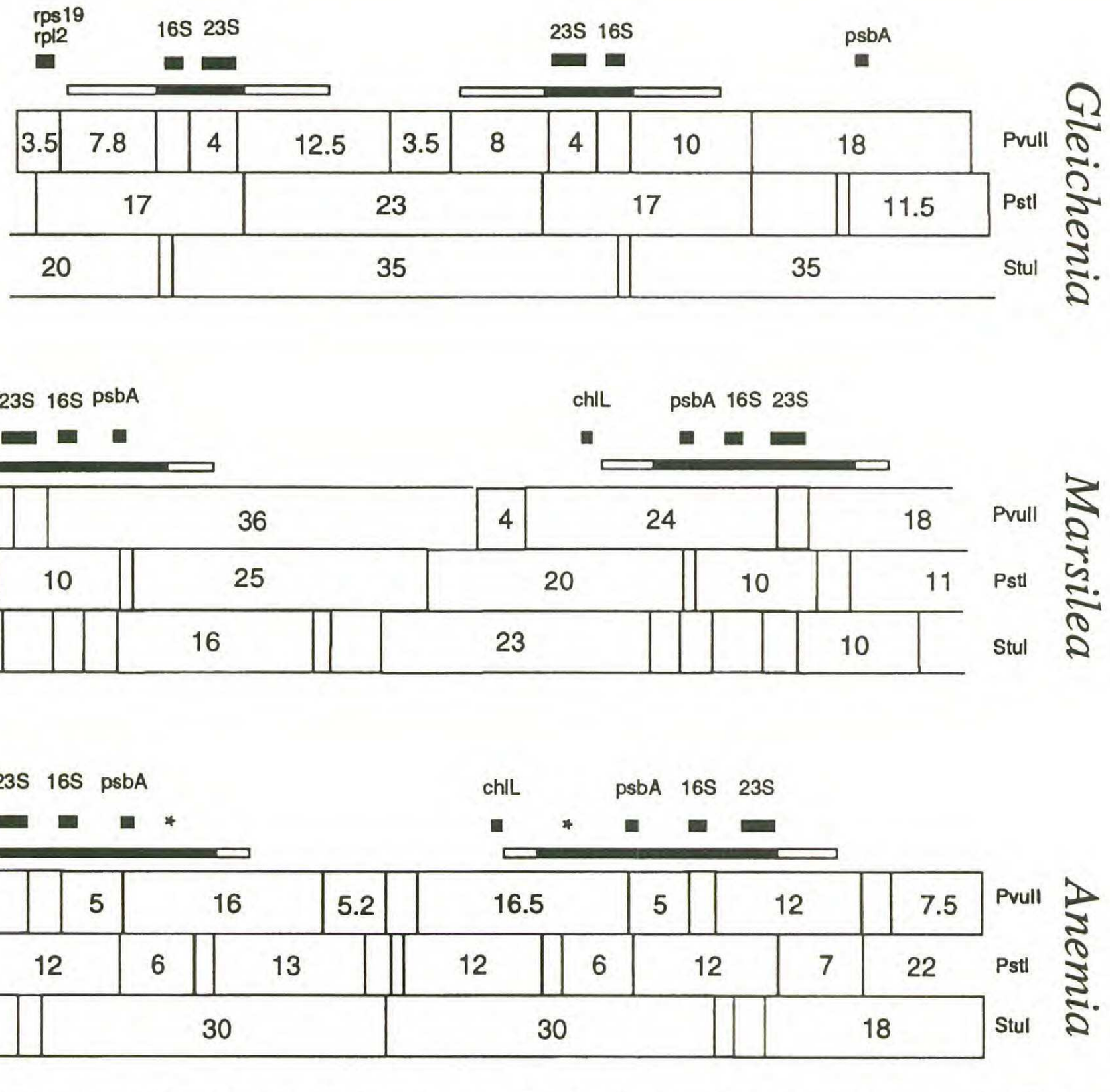

FIG. 2. Physical maps of the chloroplast genomes of *Gleichenia, Marsilea,* and *Anemia.* Shown are the IR, SSC and the adjacent LSC region for three single enzyme digests. The bar above the map denotes the extent of the IR: Minimum extent=solid; maximum extent=open. Filled boxes indicate positions of selected genes. Note the duplication of *psbA* and the change in orientation of 23S and 16S rDNA in *Marsilea* and *Anemia,* but not in *Gleichenia.* The asterisks (*) above the *Anemia* map represent the location of the extra partial *chlL* copies. Numbers represent the sizes of the fragments in kb. Sizes are not included in fragments less than 3-kb.

RESULTS

We have produced physical maps that include the SSC, the IR, and the flanking ends of the LSC for the cpDNA of *Gleichenia, Marsilea,* and *Anemia* (Fig. 2), as well as of *Schizaea, Lygodium,* and *Polypodium.* The *Gleichenia* genome is slightly changed from the *Osmunda*-like structure, with *ndhB* added to the IR. The other fern cpDNAs have a more highly modified *Adiantum*-like structure. This *Adiantum*-like condition, involving duplication of the region from *rps7* through *psbA* and two inversions in the IR, appears to be widespread in ferns. In addition to those ferns described earlier (Stein et al., 1992), the cpDNAs of *Schizaea, Anemia,* and *Lygodium, Marsilea,* and *Polypodium* are co-linear to the *Adiantum* genome in this regard (Table 1).

In preparing our maps, we determined the position of *chlL* using the 261-bp and 363-bp PCR products generated from *Polystichum acrostichoides.* The

chlL gene, which codes for one subunit of the enzyme that produces chlorophyll in the absence of light, is located in the SSC adjacent to the IR of most pteridophytes (Raubeson, 1991; Burke et al., 1993). However, in some fern cpDNAs, a portion of *chlL* is duplicated. The more inclusive 363-bp product hybridizes to an additional region of the genome that is not adjacent to the region detected by both the smaller and the larger products. The mapped location of the additional region of homology suggests that the duplicated portion of *chlL,* approximately 200-bp of the 5′ end of the gene, is not located at the IR-SSC junction. Instead it has been moved into the IR so that *trnN* is between the partially duplicated copy and the complete gene in the SSC. Thus, this portion of *chlL* occurs in two locations within the IR in addition to the complete SSC gene. Although it actually occurs three times in the genome, we will refer to it as "duplicated".

As part of our examination of the duplication of *chlL,* we extended our survey of genome structure to additional taxa with previously-published maps. We tested *Osmunda* (Palmer and Stein, 1982, 1986; Raubeson, 1991), *Polystichum* (Burke et al., 1993), *Dryopteris* (Hutton, 1992), and a variety of tree ferns including *Lophosoria* and *Dicksonia,* as well as more members of the Cyatheaceae (Conant et al., 1994) by hybridization of the two *chlL* PCR products to filters prepared for these earlier studies. All but *Osmunda* contained the duplicated and relocated gene fragment. In addition, we prepared a dot blot of cloned *Adiantum* cpDNA to determine that the *chlL* duplication and relocation occurs in *Adiantum* (Fig. 3). Also, we prepared filters with *Pteridium* DNA and the enzymes used in preparing the previously-published maps (Tan and Thompson, 1990). The 363-bp product detected an additional non-adjacent region of the *Pteridium* genome relative to the smaller 261-bp fragment.

In a few instances, we are not able to confirm that the duplicated portion is internal to the IR. Restriction fragments serve as our reference points; if recognition sites for the enzymes that we used were not appropriately positioned we could not differentiate between a duplication at the IR/SSC junction and a duplicated portion that moved within the IR. These two locations are only about 4-kb apart and a restriction site must occur between them to distinguish between these two possibities. However, whenever we could make a determination, the duplicated portion was always relocated. The pattern, of a partially duplicated and (definitively or presumably) relocated piece of *chlL,* is found in all of the fern cpDNAs that possess the *psbA* duplication and two inversions except possibly *Marsilea* (Table 1). In *Marsilea* (as in *Osmunda* and *Gleichenia*) there was no difference in hybridization pattern between the two PCR products.

Discussion

The ancestral IR gene complement consisting of the rDNA operon is observed in the cpDNAs of *Marchantia* (Ohyama et al., 1986), *Selaginella, Equisetum, Botrychium* and *Osmunda* (Raubeson, 1991). When the IR of vascular plant cpDNAs contain additional genes, *rps7,12* and *ndhB* genes are next in-

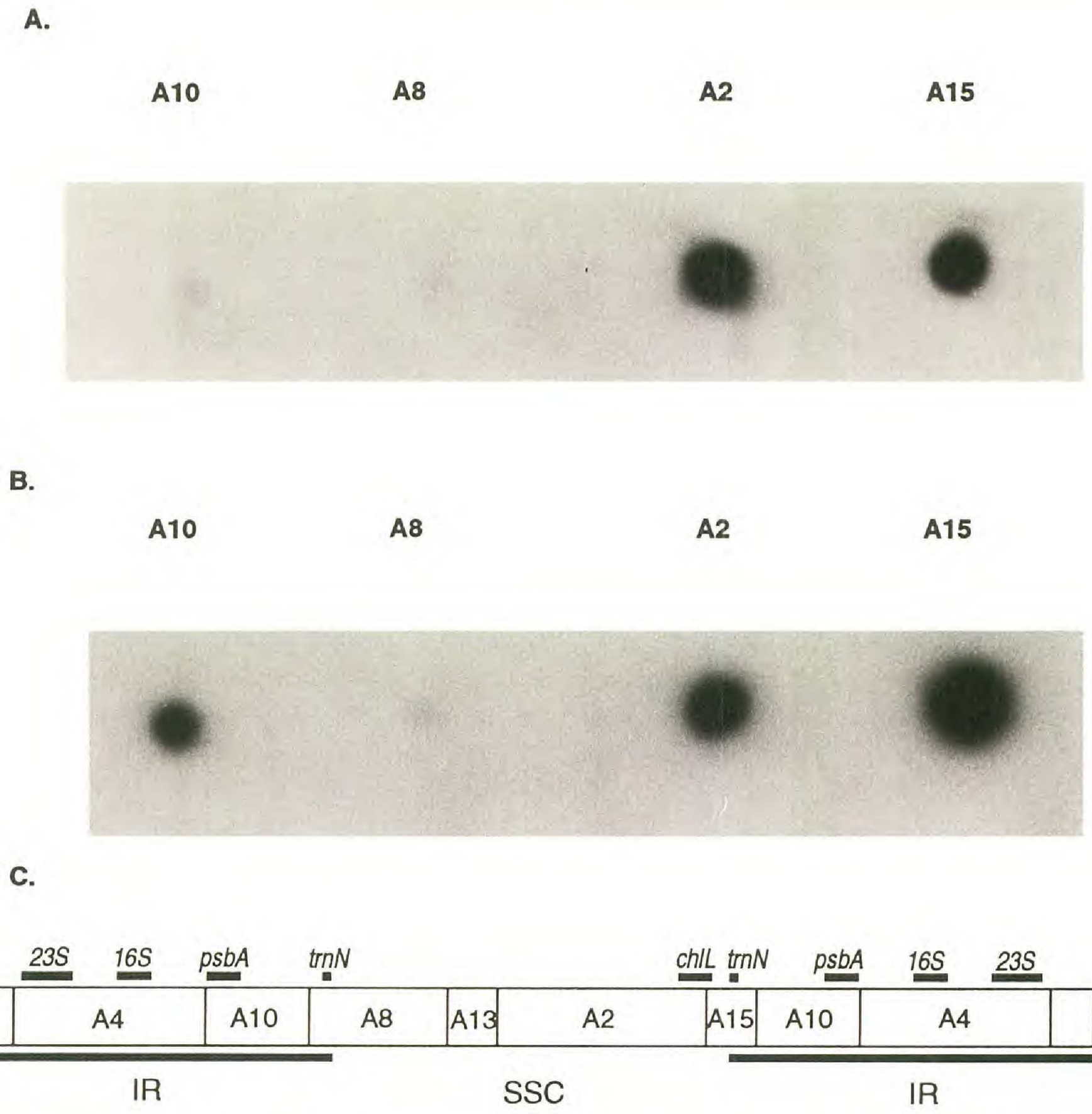

FIG. 3. Results of hybridizing the *chlL* PCR products to cloned cpDNA fragments from *Adiantum capillus-veneris*. A) The 261-bp *chlL* product hybridized to a dot blot of four of the cloned fragments. B) Results from a hybridization of the 363-bp product to the same dot blot filter. C) Physical map of the cloned fragments through the IR and SSC regions; A10 is completely in the IR, A15 and A8 include the IR/SSC junction, and A2 is completely in the SSC region. Note that A2 and A15 show a signal with both probes whereas A8 does not hybridize with either. This indicates that the complete copy of *chlL* is not in the duplicated (IR) portion of A15 as there is no homologous sequence in A8. The 363-bp probe (B), but not the 261-bp probe (A), has hybridized to A10. This indicates that there is a portion of A10 homologous to the unique portion of the 363-bp probe, presumably the partial copy of *chlL,* and it is not adjacent to the IR/SSC junction.

cluded sequentially (Raubeson, 1991; Raubeson, Stein, and Conant, 1995). In *Gleichenia,* the IR contains *ndhB* but not *rps7,12,* indicating that an inversion (that reversed the order of *rps7,12* and *ndhB*) has taken place in addition to a duplication. Both the duplication and the inversion of these genes may be unique to *Gleichenia.* Alternatively, the duplication may represent a stage intermediate between the ancestral genome structure, as found in *Osmunda,* and the *Adiantum*-like condition. Regardless of how its IR arrangement evolved, *Gleichenia* clearly lacks the complex rearrangements found in the cpDNA of *Adiantum* and many other ferns.

The majority of fern cpDNAs examined share the derived *Adiantum*-like genome structure—the rearrangement pattern that involves the duplication of several genes and two large inversions within the inverted repeat (Fig. 4). Gene duplications of this type are relatively uncommon and inversions within the IR are very rare. A minimum of three independent events, each with a low probability of occurrence, must have combined to give the observed structure. This series of events is highly unlikely to have occurred in parallel or to revert to the ancestral condition. The widespread distribution of this complex of characters indicates that the majority of extant leptosporangiate ferns share a unique common ancestor. Of those ferns studied so far, only the *Osmunda* and *Gleichenia* lineages diverged prior to the existence of this ancestral fern.

Our results conflict with various proposals that extant ferns have arisen as multiple lineages that pre-date the divergence of *Osmunda.* Numerous such phylogenies have been proposed, including Mickel (1974) and Holttum (1973), as well as Mehra (1961), Ching (1940), and others influenced by Bower's (1923–1928) evolutionary ideas based on soral position. Other treatments, however, have proposed phylogenetic scenarios that include all the extant leptosporangiate ferns as a single clade. The Osmundaceae, with many morphological features intermediate between eusporangiate and the other leptosporangiate ferns, are commonly regarded as the basal extant lineage of leptosporangiate ferns (Wagner, 1989; Bierhorst, 1971; Nayar, 1970). The cpDNA structural data support this placement of *Osmunda* as do other molecular data sets such as *rbcL* sequences (Hasebe et al, 1994) and preliminary analyses of 18S rDNA (Raubeson and Stein, unpublished data). The structural data also support the placement of *Gleichenia* at the base of the fern phylogeny. (At present our data do not resolve the relative placement of *Osmunda* and *Gleichenia.*) *Gleichenia,* due to the "stem-like" nature of the leaves, a potentially long fossil record, and other features, has commonly been regarded as a basal lineage (Bierhorst, 1971; Bower, 1923–28; and numerous others). The *rbcL* analysis (Hasebe et al., 1994) also supports the divergence of *Gleichenia* after *Osmunda* but prior to any of the other ferns examined here. Some pteridologists (Holttum, 1973; Bierhorst, 1971; Nayar, 1970) have contemplated a possible derivation of the Polypodiaceae from within the gleicheniaceous lineage. The cpDNA structural data are in conflict with that hypothesis.

In addition to the *psbA* duplication and two inversions, cpDNAs of *Adiantum* and other higher leptosporangiate ferns possess another rearrangement, the partial duplication of *chlL.* Thus far we have documented this character only through Southern hybridization with the two PCR products. Obviously, lack of an additional, separate hybridization signal with the more inclusive fragment does not necessarily mean that the duplication did not occur. If the duplicated portion has not been conserved, sequence divergence could result in our inability to detect the region by this method. A secondary loss of the duplication could also occur by deletion. However, our interpretation at present is that the lack of an extra region of homology results from a (primary) lack of the duplication. Presumably, the duplication occurred initially through growth of the IR, which copied a portion of the *chlL* gene to the other IR/SSC

FIG. 4. Diagrammatic representation of a possible scenario that would explain the structural changes detected in fern cpDNAs. The linear representation shows the two copies of the IR, the intervening SSC and the adjacent portions of the LSC region. The boxes represent genes, above or below the line indicating orientation of the coding strand. Solid boxes are duplicated genes in the IR and open boxes are single copy genes. Not all single-copy genes are shown, and the SSC region is not drawn to scale. The size of the boxes is proportional to the size of the gene. Shown are five stages of fern cpDNA evolution: 1) the ancestral state as found in *Osmunda*; 2) hypothetical subsequent stage (that would then be further, and independently, modified to result in the *Gleichenia* condition) resulting from (a) duplication of *rps7/12* and *ndhB*; 3) hypothetical stage involving an inversion (b) that reverses the region between *ndhB* and *psbA* and is (c) subsequently incorporated into the other copy of the IR; 4) the stage seen in *Marsilea*, resulting from an additional inversion (d) that reverses a large portion of the IR; 5) the condition found in the majority of fern cpDNAs resulting from the duplication of a portion of *chlL* (e), the relocation of that duplicated portion (f), and the copy correction of the relocated piece into the other copy of the IR (g). [Genes shown in the diagram: *rpl2*, *rps7/12*=ribosomal protein genes; 16S and 23S=small and large subunits of the plastid ribosomes; R and N=tRNA genes; *chlL*=subunit of light-independent protochlorophyllide reductase; *ndhB*=subunit of NADH dehydrogenase; orf=open reading frame (putative gene of unknown function); *psbA*=photosystem II 32-kD protein.]

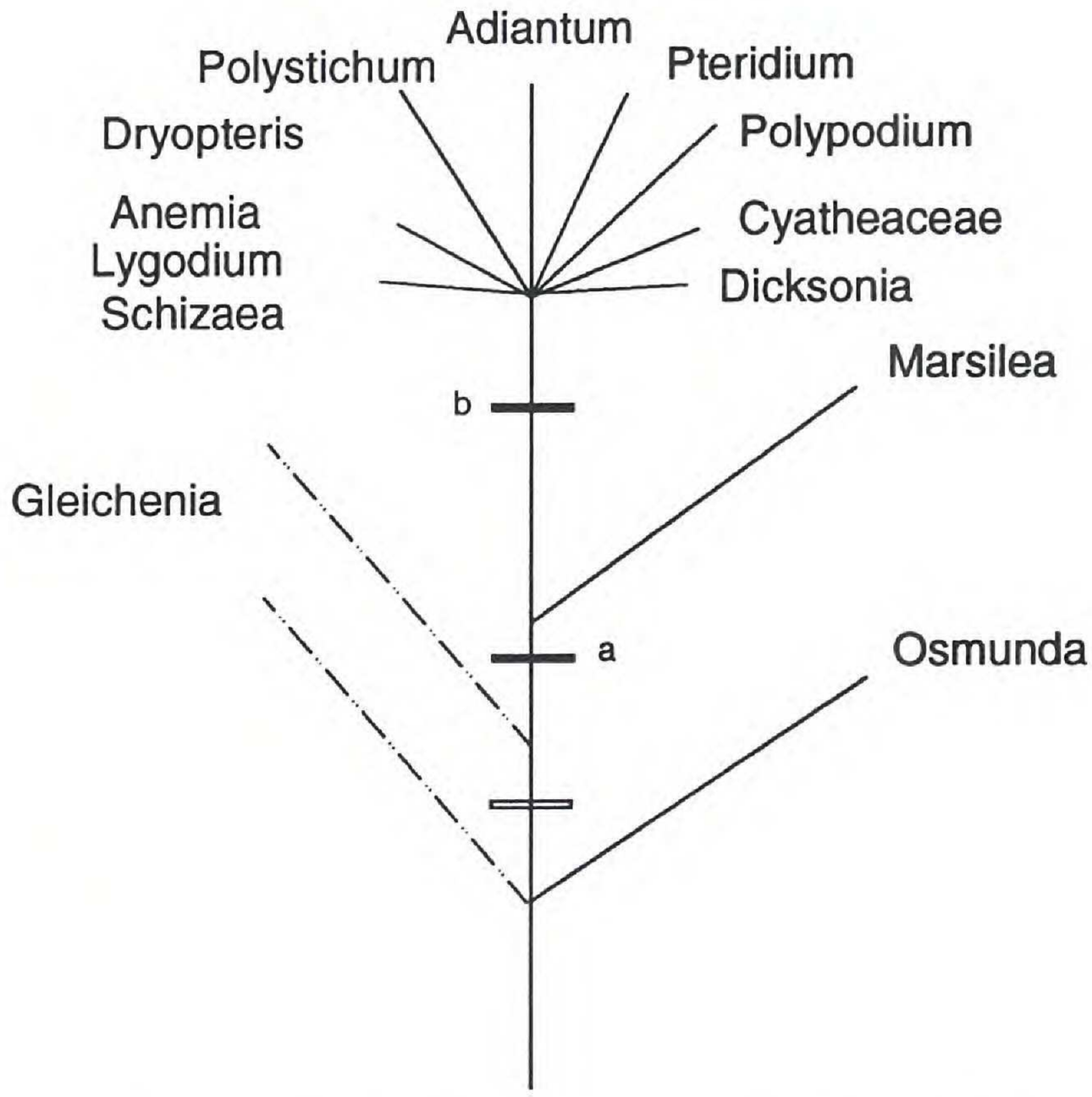

FIG. 5. Fern phylogeny supported by the cpDNA structural mutations. The open bar represents the potentially-shared duplication of *rps12,7* and *ndhB*. The position of *Gleichenia* (the dashed branches) varies according to the interpretation of the IR gene content. Bar a represents the *psbA* duplication and two inversions. Bar b represents the partial duplication and movement of *chlL*.

junction. The nature of the change that led to the partial copy being positioned away from the junction is unclear. A small inversion seems unlikely because sequence data from *Polystichum acrostichoides* show that the sequence between the complete copy of *chlL* and one of the partial copies is in its usual (rather than the inverted) orientation (Burke et al., 1993). One possible explanation for the relocated, non-inverted partial copy would be transposition. However this mechanism has not been demonstrated in the chloroplast genome. We are currently pursuing a sequencing strategy to clarify the nature and distribution of this feature.

Marsilea, although sharing the *psbA* duplication and two inversions, may lack the *chlL* rearrangement. Marsileaceous ferns have been difficult to place in traditional phylogenetic schemes because of their highly derived morphology. Many authors omit them from evolutionary treatments (e.g., Holttum, 1974; Mickel, 1973; Nayar, 1970). When included, the Marsileaceae are usually most closely allied to the Schizaeaceae (e.g., Wagner, 1989; Bierhorst, 1971; Mehra, 1961). The distribution of the *chlL* rearrangement conflicts with this hypothesis. However, as Tryon and Tryon (1982) have noted, "many of the characters that have been used to support the relationship of the two families [Marsileaceae and Schizaeaceae], . . . are characters of primitive leptosporangiate ferns rather than those especially of the Schizaeaceae." The cpDNA structural data suggest that *Marsilea* diverged from the main branch of fern evolu-

tion after *Osmunda* and *Gleichenia* but before the origin of most higher ferns, including the Schizaeaceae (Fig. 5).

Although cpDNA structural changes will not be able to resolve all questions of fern relationships, they help to clarify some fundamental aspects of fern phylogeny. The results presented here make powerful phylogenetic statements and indicate to us that additional taxa should be characterized to gather data on the placement of other putatively basal ferns. Of special interest will be *Salvinia,* a representative of the other group of heterosporous water ferns (sister-group to the Marsileales in the *rbcL* tree but more commonly regarded as a quite disparate lineage), *Dipteris, Cheilopleuria,* and *Hymenophyllum,* as well as *Stromatopteris* and *Psilotum* (in light of Bierhorst's (1977) suggestion that *Psilotum* is a reduced leptosporangiate fern and sister taxon of the gleicheniaceous ferns). These studies are now underway.

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